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Reevaluation of an *Acanthamoeba* Molecular Diagnostic Algorithm following an Atypical Case of Amoebic Keratitis

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Amoebic keratitis (AK) is a potentially blinding infection, the prompt diagnosis of which is essential for limiting ocular morbidity. We undertook a quality improvement initiative with respect to the molecular detection of acanthamoebae in our laboratory because of an unusual case of discordance. Nine ATCC strains of *Acanthamoeba* and 40 delinked, biobanked, surplus corneal scraping specimens were analyzed for the presence of acanthamoebae with four separate real-time PCR assays. The assay used by the Free-Living and Intestinal Amebas Laboratory of the CDC was considered the reference standard, and the performance characteristics of each individual assay and pairs of assays were calculated. Outcome measures were sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV). Of 49 included specimens, 14 (28.6%) were positive by the gold standard assay, and 35 (71.4%) were negative. The sensitivities of the individual assays ranged from 64.3% to 92.9%, compared to the gold standard, while the specificities ranged from 88.6% to 91.4%. The PPVs and NPVs ranged from 69.2% to 78.6% and from 86.1% to 96.9%, respectively. Combinations of assay pairs led to improved performance, with sensitivities ranging from 92.9% to 100% and specificities ranging from 97.1% to 100%. ATCC and clinical strains of *Acanthamoeba* that failed to be detected by certain individual assays included *Acanthamoeba castellanii*, *Acanthamoeba culbertsoni*, and *Acanthamoeba lenticulata*. For three clinical specimens, false negativity of the gold standard assay could not be excluded. Molecular diagnostic approaches, especially combinations of highly sensitive and specific assays, offer a reasonably performing, operator-independent, rapid strategy for the detection of acanthamoebae in clinical specimens and are likely to be more practical than either culture or direct microscopic detection.

Amoebic keratitis (AK) is a potentially blinding eye infection caused by the parasite *Acanthamoeba*, which is a ubiquitous, free-living organism found in soil and other environmental sources (1). This infection usually occurs in the context of contact lens use, and outbreaks of AK have been linked to contact lens solutions that are inefficient in killing acanthamoebae adhering to the contact lenses during washing with ameba-contaminated water, including the most recent outbreak in the United States, which affected 138 people (2) and led to the recall of several contact lens solutions and products by both the FDA and Health Canada (3, 4). Delays in diagnosis have been associated frequently with poor visual outcomes and more severe clinical progression in AK (5, 6). Traditional diagnostic procedures include direct microscopic examination of corneal scrapings or contact lens fluids stained with Giemsa stain, periodic acid-Schiff stain, hematoxylin and eosin, or acridine orange and culture of specimens on nonnutrient agar overlaid with *Escherichia coli* or *Klebsiella pneumoniae*; both methods are limited by poor sensitivity, operator dependence, and, in the case of culture, long turnaround times (5, 6). As in all areas of clinical microbiology, molecular methods, including PCR, are quickly supplanting traditional techniques for the detection of acanthamoebae, due to superior sensitivity, standardized analytical procedures, and rapid turnaround (7–10). We validated previously two molecular approaches for the diagnosis of amoebic keratitis in our laboratory (8, 9), and here we illustrate a reevaluation process that we undertook as a quality improvement initiative following a false-negative result in our laboratory.

CASE REPORT

An agar plate that had been inoculated with a corneal scraping at a peripheral hospital and contained numerous spherical cystic bodies characteristic of a free-living amoeba (Fig. 1) was received in our reference laboratory and examined by standard light microscopy. Due to the absence of a wrinkled exocyst and polyhedral endocyst morphology (Fig. 2), as well as an atypical growth pattern on agar (Fig. 3 and 4), we performed our standard real-time PCR assay that is specific for *Acanthamoeba* spp., as described previously (9), which yielded negative results on 3 consecutive occasions. Due to this discrepancy, we submitted the agar plates to the Free-Living and Intestinal Amebas (FLIA) Laboratory at the Centers for Disease Control and Prevention (CDC) (Atlanta, GA) for additional reference examination. Examination at the CDC FLIA Laboratory confirmed mor-

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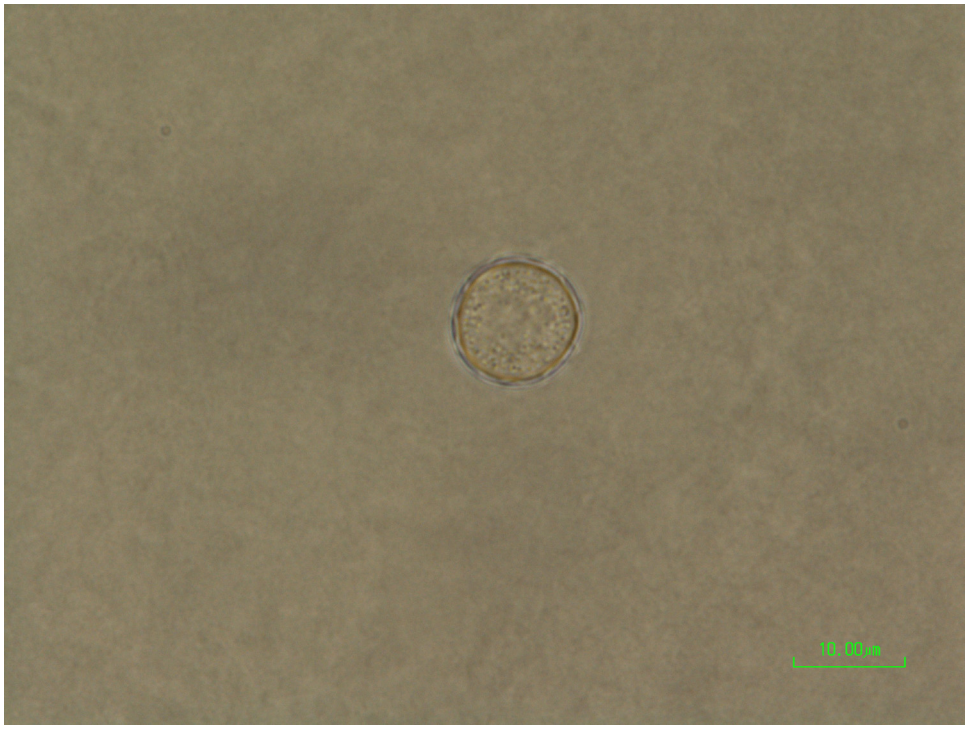

FIG 1 Atypical amoeboid cystic bodies on an agar plate received in our reference laboratory. Unstained, ×1,000 magnification.

phology consistent with an *Acanthamoeba* sp., and a triplex real-time PCR assay for the detection of *Naegleria fowleri*, *Balamuthia mandrillaris*, and *Acanthamoeba* spp. (10) yielded positive results for *Acanthamoeba* spp., with threshold cycle (C_T) values of 25.9 and 28.1 with 5 μl and 1 μl of DNA per reaction, respectively. We then undertook a quality improvement initiative to identify the source of the nonconformance in our laboratory.

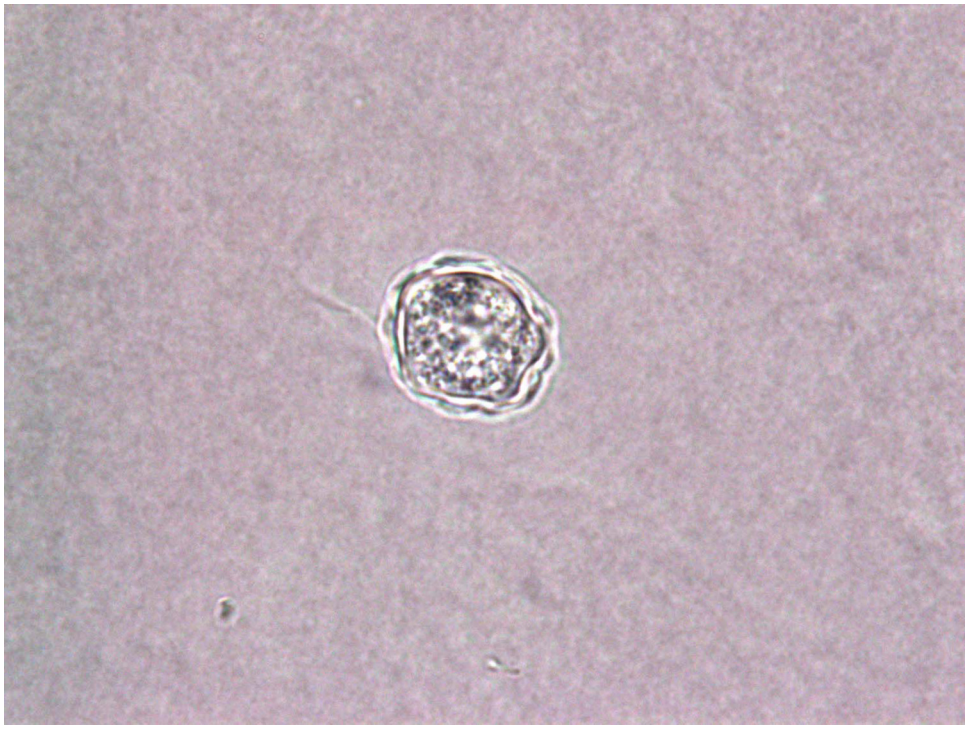

FIG 2 Wrinkled exocyst and polyhedral endocyst morphology typical of *Acanthamoeba* cysts. Unstained, ×1,000 magnification.

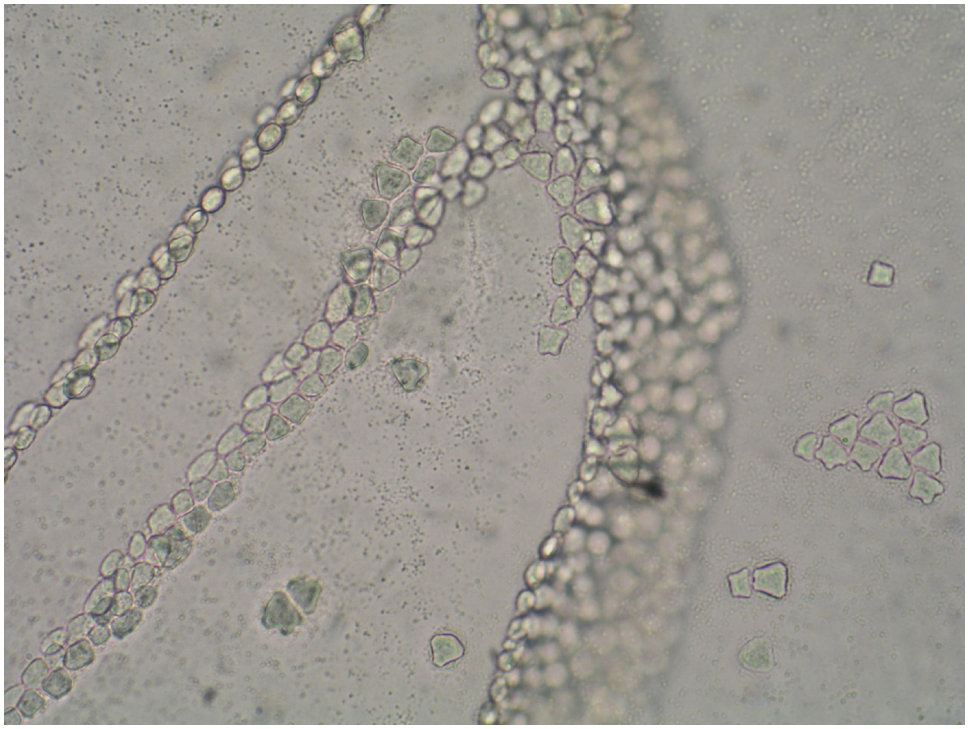

FIG 3 Typical growth pattern of *Acanthamoeba* cysts in nonnutrient agar overlaid with *Klebsiella* spp. or *E. coli*. Note the presence of contiguous sheets of cysts. Unstained, ×100 magnification.

MATERIALS AND METHODS

Specimens. Delinked surplus and biobanked corneal scrapings stored at −80°C between 1 January 2015 and 17 April 2015 were identified and retrieved. In addition, we retrieved 9 banked ATCC strains of *Acanthamoeba* spp., i.e., *Acanthamoeba castellanii* (Douglas) Page (ATCC 50373, 50493, and 50739), *Acanthamoeba castellanii* (ATCC 50492), *Acanthamoeba culbertsoni* Singh and Das (ATCC 30171), *Acanthamoeba polyphaga* (Puschkarew) Page (ATCC 30173, 50372, and

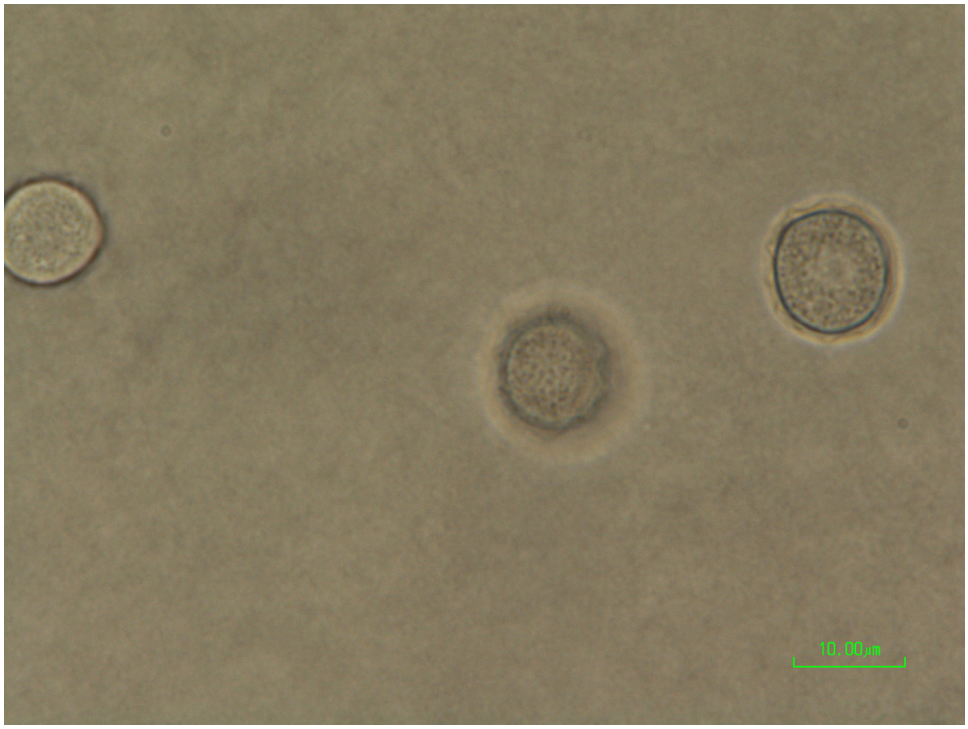

FIG 4 Atypical growth pattern of isolated amoeboid cystic bodies on an agar plate received in our reference laboratory. These cysts were later confirmed to be *Acanthamoeba lenticulata*. Unstained, ×1,000 magnification.

50495), and *Acanthamoeba* sp. (ATCC PRA-220), to serve as positive-control specimens.

Selection of tests for validation. We searched Medline from 2006 to 13 April 2015, using combinations of the following search terms: "*Acanthamoeba*," "acanthamoebae," and "amoebic keratitis" with "diagnostic sensitivity" and "test performance." We restricted the search to English-language papers and to studies conducted with humans. We selected 4 different primer sets to evaluate, namely, those described by Riviere and colleagues (11), those described by Qvarnstrom and colleagues (10), and the 2 sets of primers initially validated for clinical use in our laboratory (Nelson and JDP primers) (8, 12, 13).

DNA extraction. Primary samples were subjected to three freeze-thaw cycles with liquid nitrogen and a 56°C heat block, to disrupt *Acanthamoeba* cysts. DNA was then extracted with the DNA minikit tissue protocol (Qiagen, Germantown, MD) and eluted with 60 μl of buffer AE (10 mM Tris-Cl, 0.5 mM EDTA; pH 9.0). DNA was stored at −20°C prior to use.

Real-time PCR. Four independent real-time PCR assays targeting the 18S rRNA region of *Acanthamoeba* spp. were compared, including two TaqMan (referred to as Riviere and Qvarnstrom assays) and two Sybr green (referred to as Nelson and JDP assays) real-time PCR assays, as described previously (9–13). The Riviere assay was performed with 200 nM each forward (5′-CGACCAGCGATTAGGAGACG-3′) and reverse (5′-CCGACGCCAAGGACGAC-3′) primers and 100 nM probe (5′-6-carboxyfluorescein [FAM]-TGAATACAAAACACCACCATCGGCGC-6-carboxytetramethylrhodamine [TAMRA]-3′) (11). The Qvarnstrom assay was performed with 400 nM each forward (5′-CCCAGATCGTTTA CCGTGAA-3′) and reverse (5′-TAAATATTAATGCCCCCAACTATCC-3′) primers and 200 nM probe (5′-FAM-CTGCCACCGAATACATTAG CATGG-black hole quencher 1 [BHQ1]-3′) (10). Both assays included amplification with 1× Universal TaqMan master mix (Life Technologies catalog no. 4304437) and 5 μl of DNA, in a total volume of 25 μl, with the following cycling conditions: 50°C for 2 min, 95°C for 10 min, and 45 cycles of 95°C for 15 s and then 60°C (Riviere assay) or 55°C (Qvarnstrom assay) for 1 min. The Nelson assay was performed with 400 nM each forward (5′-GTTTGAGGCAATAACAGGT-3′) and reverse (5′-GAATT CCTCGTTGAAGAT-3′) primers (13) and the JDP assay was performed with 400 nM each forward (5′-GGCCCAGATCGTTTACCGTGAA-3′) and reverse (5′-TCTCACAAGCTGCTAGGGAGTCA-3′) primers (12) in 1× Power Sybr green master mix (Life Technologies catalog no. 4365877) with 5 μl of DNA, in a total volume of 25 μl. Both assays used cycling conditions of 95°C for 10 min, 45 cycles of 95°C for 15 s and then 60°C for 1 min, and a dissociation step. All real-time PCR assays were conducted in an ABI 7900HT fast real-time PCR system. Any positive amplification with the Sybr green assay was verified by running 10 μl of PCR product on a 1% agarose gel at 100 V for 30 min, in the presence of ethidium bromide.

Species identification by sequencing. For the atypical specimen that was received in our laboratory and triggered the quality improvement initiative, sequence BLAST analysis of the PCR product from each primer set was performed for maximal sequence homology determination. Sanger sequencing was performed with PCR products that had been purified with the QIAquick PCR purification kit (Qiagen, Germantown MD), using the same forward and reverse primers with the BigDye Terminator v3.1 cycle sequencing kit (Life Technologies), according to the manufacturer's instructions. Sanger-sequenced products were purified with a BigDye Xterminator purification kit (Life Technologies) and analyzed with an ABI 3130xl genetic analyzer.

Performance characteristics and reference standard. We considered a specimen positive if the reference assay used by the FLIA Laboratory at the CDC (10) produced a logarithmic amplification curve with a C_T value of ≤45. We considered a specimen negative if the reference assay used by the FLIA Laboratory at the CDC (10) failed to produce logarithmic amplification or yielded a C_T value of >45. We calculated routine performance characteristics, including sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV), for our current primer set (9, 11) and the JDP and Nelson primers (8, 12, 13). We also calculated the performance characteristics of a composite reference standard, for which agreement of at least 2 of 3 tests (1 test had to be the Qvarnstrom assay [10]) was considered definitive (i.e., a true-positive or true-negative result). In this way, the performance characteristics of the Riviere-JDP combination (11, 12), the Riviere-Nelson combination (11, 13), and the Nelson-JDP combination (12, 13) were assessed.

TABLE 1 Performance characteristics of 3 assays for molecular detection of *Acanthamoeba* spp. in 40 surplus clinical specimens and 9 ATCC positive-control strains

Primer Set	No. Positive	No. Negative	Sensitivity (%)	Specificity (%)	PPV[a] (%)	NPV (%)
Qvarnstrom[b] (10)	14	35	NA	NA	NA	NA
Riviere (11)	14	35	78.6	91.4	78.6	91.4
Nelson (8,13)	13	36	64.3	88.6	69.2	86.1
JDP (8,12)	16	33	92.9	91.4	81.3	97.0

[a] PPV, positive predictive value; NPV, negative predictive value; NA, not applicable.
[b] Reference standard used by the Free-Living and Intestinal Amebas laboratory of the Centers for Disease Control and Prevention.

RESULTS

Between 1 January 2015 and 17 April 2015, 40 surplus corneal specimens submitted to the clinical parasitology laboratory for detection of *Acanthamoeba* spp. were biobanked; those specimens were included in this analysis. All 9 ATCC positive-control specimens were positive by the CDC FLIA Laboratory assay. Of the 40 biobanked specimens, 5 (12.5%) were positive by the CDC FLIA Laboratory assay (10) and were considered true-positive samples for the purposes of our validation. Thirty-five of the 40 biobanked specimens (87.5%) were negative by the CDC FLIA Laboratory assay (10) and were considered true-negative samples for the purposes of our validation.

Of 49 total specimens (40 biobanked clinical specimens and 9 ATCC strains of *Acanthamoeba* spp.), 14 (28.6%) were positive (8/9 ATCC strains and 6/40 clinical specimens) and 35 (71.4%) were negative (1/9 ATCC strains and 34/40 clinical specimens) with the Riviere assay (11) (Table 1), yielding a sensitivity of 78.6% and a specificity of 91.4%. The PPV and NPV were 78.6% and 91.4%, respectively, compared to the reference standard. Two false-negative specimens with this assay were identified as *A. culbertsoni* (ATCC 30171) and *A. lenticulata*.

Of 49 total specimens, 13 (26.5%) were positive (7/9 ATCC strains and 6/40 clinical specimens) and 36 (73.5%) were negative (2/9 ATCC strains and 34/40 clinical specimens) with the Nelson assay (8, 13), yielding a sensitivity of 64.3% and a specificity of 88.6% (Table 1). The PPV and NPV were 69.2% and 86.1%, respectively, compared to the reference standard. One false-negative specimen with this assay was identified as *A. polyphaga* (ATCC 50495) and another as *A. castellanii* (ATCC 50373).

Of 49 total specimens, 16 (32.7%) were positive (9/9 ATCC strains and 7/40 clinical specimens) and 33 (67.3%) were negative (0/9 ATCC strains and 33/40 clinical specimens) with the JDP assay (8, 12), yielding a sensitivity of 92.9% and a specificity of 91.4% (Table 1). The PPV and NPV were 81.3% and 97.0%, respectively, compared to the reference standard.

Using the Qvarnstrom (CDC) assay (10) as the arbiter test, the combination of the Riviere (11) and JDP (8, 12) assays yielded 100% sensitivity and 97.1% specificity, with a PPV and a NPV of 93.3% and 100%, respectively (Table 2). The combination of the

TABLE 2 Performance characteristics of combinations of 3 assays for molecular detection of *Acanthamoeba* spp. in 40 surplus clinical specimens and 9 ATCC positive-control strains

Primer Set	No. Positive	No. Negative	Sensitivity (%)	Specificity (%)	PPV[a] (%)	NPV (%)
Qvarnstrom[b] (10)	14	35	NA	NA	NA	NA
Riviere (11) + JDP (8,12)	15	34	100.0	97.1	93.3	100.0
Riviere (11) + Nelson (8,13)	13	36	92.9	100.0	100.0	97.2
Nelson (8,13) + JDP (8,12)	13	36	92.9	100.0	100.0	97.2

[a] PPV, positive predictive value; NPV, negative predictive value; NA, not applicable.
[b] Reference standard used by the Free-Living and Intestinal Amebas laboratory of the Centers for Disease Control and Prevention.

Riviere (11) and Nelson (8, 13) assays yielded 92.9% sensitivity and 100% specificity, with a PPV and a NPV of 100% and 97.2%, respectively, compared to the Qvarnstrom assay (10); discrepant results were arbitrated with the Qvarnstrom assay (Table 2). The combination of the Nelson (8, 13) and JDP (8, 12) assays resulted in a sensitivity of 92.9%, a specificity of 100%, a PPV of 100%, and a NPV of 97.2%, compared to the Qvarnstrom assay (Table 2).

For 9 clinical specimens that were deemed negative with the Qvarnstrom assay, one other assay was positive with a C_T value of ≤45. For one specimen, both the Riviere and JDP assays were positive, although these specimens were called false-positive specimens for the purposes of this analysis, due to the designation of the Qvarnstrom assay as the gold standard. Isolated Riviere assay positivity occurred for 2 of 9 specimens that were negative by the Qvarnstrom assay, while isolated positivity with the Nelson and JDP assays occurred for 4 and 2 specimens, respectively. For one specimen, both the Riviere and JDP assays were positive. For the specimens with isolated Nelson and JDP assay positivity ($n = 7$ specimens), gel electrophoresis confirmed those to be false-positive results, due to incompatible band sizes.

Sequence BLAST analysis of the positive PCR products from the Qvarnstrom (10), Nelson (8, 13), and JDP (8, 12) assays for the atypical case that triggered our investigation revealed 100% homology with *Acanthamoeba lenticulata*.

DISCUSSION

Amoebic keratitis is a potentially blinding protozoal infection, the diagnosis of which depends on microscopic demonstration of acanthamoebae directly from clinical specimens, such as corneal scrapings, or by culture or molecular detection of genomic targets. Culture and direct examination are limited by poor sensitivity, the need for technical expertise, and long turnaround times, while molecular diagnostic methods offer the advantages of rapidity, sensitivity, and operator independence (5–8). Several years ago, our laboratory selected the Riviere assay (11) as our molecular diagnostic approach, which appeared to offer the best possible limit of detection for acanthamoebae from clinical specimens (9, 11) and was thus thought to be highly sensitive for the *Acanthamoeba* genus. However, an atypical case of *A. lenticulata* (a rare cause of AK [14]) that presented to our laboratory highlighted deficiencies in the ability of the Riviere primer set (11) to detect this species. In addition, we found that the Riviere primer set (11) failed to identify an ATCC strain of *A. culbertsoni*, which is not an unusual cause of AK (15).

The Nelson assay (8, 13) failed to detect one ATCC strain of *A. castellanii* and one strain of *A. polyphaga*, whereas the JDP (8, 12) and Qvarnstrom (10) assays detected all 9 ATCC strains of *Acanthamoeba*. Although we considered the Qvarnstrom assay (10) to be the gold standard in this analysis, it should be recognized that even this reference test may have subtle performance limitations. Although most cases of isolated Riviere (11), Nelson (8, 13), or JDP (8, 12) assay positivity were confirmed to be false-positive results, due to the superior and previously published limit of detection of the Riviere assay (9, 11), we cannot exclude the possibility of false negativity of the Qvarnstrom assay in 3 cases.

The limitations of any one assay versus the others appeared to be corrected through the use of a composite reference standard, for which agreement of at least 2 of 3 tests (1 test had to be the Qvarnstrom assay [10]) led to >95% sensitivity and specificity. The combination of the Riviere (11) and JDP (8, 12) assays appeared to offer the best performance characteristics when arbitrated by the Qvarnstrom assay (10), although our study was not powered to detect statistically significant differences in performance among the combinations of assays. Given that no single molecular assay offers perfect performance, it seems prudent to perform at least two (if not three) assays, either sequentially or simultaneously, in order to improve the sensitivity and specificity, considering the grave prognosis of untreated AK. Use of the composite reference standard approach ensured that all 9 ATCC strains of *Acanthamoeba*, as well as the unusual *A. lenticulata* clinical case, were detected, and it reduced individual false positivity and false negativity rates. We have now amended our approach in the laboratory to test all specimens initially with the Riviere assay (11) and then to confirm negative results using both the Qvarnstrom (10) and JDP (8, 12) assays. Optimization of this approach into a single triplex assay warrants future consideration.

Limitations of this analysis include the lack of true parasitological confirmation by culture or direct examination and the use of a surrogate gold standard method, as well as the relatively small number of surplus specimens biobanked, as expected for corneal scrapings. Because this analysis arose from a retrospective examination for quality improvement purposes, the study was not powered to detect statistical differences in the performance characteristics of the assays, and we did not attempt any such statistical maneuvers.

In summary, although culture-based diagnosis and direct microscopic examination of clinical specimens for acanthamoebae offer the greatest possible specificity for the diagnosis of AK, practical limitations of these tests, including waning technical expertise and poor turnaround times, reduce their utility in clinical parasitology. Molecular diagnostic approaches, especially the use of combinations of highly sensitive and specific assays, offer a reasonably performing, operator-independent, rapid strategy for the detection of acanthamoebae in clinical specimens.

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We declare that we have no competing interests to disclose.

R.L., M.C., J.J., I.K.M.A., A.C.-K., J.G., J.T., and F.R. contributed to data collection, analysis, and interpretation and to writing of the manuscript. A.K.B. conceived the study, contributed to data collection, analysis,

and interpretation, and was primarily responsible for writing the manuscript. All authors critically revised the manuscript.

The findings and conclusions in this article are those of the authors and do not necessarily represent the official position of the U.S. Centers for Disease Control and Prevention.

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